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Sources of glucose production in cirrhosis by ^2H_2O ingestion and ^2H NMR analysis of plasma glucose

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Abstract

Plasma glucose ^2H enrichment was quantified by ^2H NMR in patients with cirrhosis ($n=6$) and healthy subjects ($n=5$) fasted for 16 h and given ^2H_2O to ~0.5% body water. The percent contribution of glycogenolysis and gluconeogenesis to glucose production (GP) was estimated from the relative enrichments of hydrogen 5 and hydrogen 2 of plasma glucose. Fasting plasma glucose levels were normal in both groups (87 ± 7 and 87 ± 24 mg/dl for healthy and cirrhotic subjects, respectively). The percent contribution of glycogen to GP was smaller in cirrhotics than controls (22 ± 7% versus 46 ± 4%, $P<0.001$), while the contribution from gluconeogenesis was larger (78 ± 7% versus 54 ± 4%, $P<0.001$). In all subjects, glucose 6R and 6S hydrogens had similar enrichments, consistent with extensive exchange of ^2H between body water and the hydrogens of gluconeogenic oxaloacetate (OAA). The difference in ^2H-enrichment between hydrogen 5 and hydrogen 6S was significantly larger in cirrhotics, suggesting that the fractional contribution of glycerol to the glyceraldehyde-3-phosphate (G3P)-moiety of plasma glucose was higher compared to controls (19 ± 6% versus 7 ± 6%, $P<0.01$). In all subjects, hydrogens 4 and 5 of glucose had identical enrichments while hydrogen 3 enrichments were systematically lower. This reflects incomplete exchange between the hydrogen of water and that of 1-*R*-dihydroxyacetone phosphate (DHAP) or incomplete exchange of DHAP and G3P pools via triose phosphate isomerase.

Keywords: Glucose; Deuterium; Glycogenolysis; Cirrhosis; ^2H NMR

1. Introduction

Postabsorptive glucose production (GP) is sustained by a combination of glycogen hydrolysis and gluconeogenesis. Normally, gluconeogenic flux is principally contributed by the anaplerotic pathways of the Krebs cycle with glycerol conversion to triose phosphates accounting for the remainder. In diseases such as type 2 diabetes, GP can be significantly altered due to changes in flux through one or more of the contributing pathways. This is typically accompanied by obvious clinical manifestations such as fasting hyperglycemia. However, in other cases, gluconeogenic and glycogenolytic fluxes may be modified without altering GP and glycemic state. This appears to be the case in the early stages of cirrhosis, where significant and substantial reductions in glycogenolytic flux are compensated by increased rates of gluconeogenesis [1,2]. Such changes in the sources of GP currently go unnoticed in the standard clinical setting, hence, their general significance in the pathogenesis of glucose intolerance or other disturbances of glucose homeostasis is poorly understood. Moreover, if such events represent the initial manifestations of abnormal glucose metabolism, they could provide early and, therefore, valuable markers of this condition.

To this end, the contribution of glycogenolytic and gluconeogenic fluxes to GP can now be obtained in a safe and relatively noninvasive manner by tracing the fate of deuterium from ingested ^2H_2O into the hydrogens of plasma glucose [3–5]. With this method, the contribution of glycogenolysis to GP is defined as the conversion of glycogen to glucose via the so-called 'direct pathway' (i.e. glycogen → glucose-1-phosphate → glucose-6-phos-

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phate → glucose). Glycogen that is initially metabolized to triose phosphates and then to glucose (the so-called 'indirect pathway') will experience equivalent ^2H-incorporation mechanisms to gluconeogenic substrates; hence the contribution of this pathway to GP will be included in the estimate of gluconeogenesis. The ^2H_2O procedure has been shown to be safe and well tolerated by patients and has revealed significant changes in glycogenolytic and gluconeogenic contributions to GP in several diseases including cirrhosis and diabetes [1,2,6]. The gas chromatography-mass spectrometry (GC-MS) procedure developed by Landau et al. for quantifying positional ^2H-enrichment of plasma glucose from ^2H_2O has transformed the method into a practical clinical procedure by permitting the analysis to be performed on small volumes of blood (~ 5 ml) at low levels of body water ^2H-enrichments (0.35–0.50%). However, the derivatization procedure is laborious and difficult to routinely implement in a clinical laboratory setting.

A much simpler NMR procedure for obtaining ^2H-enrichment in all positions of glucose was recently described by Schleucher et al. [7,8]. Glucose is derivatized to 1,2-*O*-isopropylidene glucose, also known as monoacetone glucose (MAG), and analyzed by ^2H-NMR under conditions where the ^2H-resonances of the glucose skeleton are fully resolved from each other and from solvent signals. This approach was used to obtain plasma glucose ^2H-enrichment information from overnight-fasted healthy subjects given ^2H_2O to ~ 0.5% body water enrichment [9]. While NMR analysis is inherently less sensitive and therefore requires a larger sample (~ 20 ml whole blood) than its MS counterpart, complete ^2H-enrichment information from plasma glucose is obtained from a simple NMR analysis of a single, easily prepared derivative.

To determine the efficacy of the ^2H_2O-NMR assay in a standard hospital setting and its potential for identifying abnormalities of endogenous glucose metabolism above and beyond that indicated by standard blood analyses, we measured and compared the fractional contribution of glycogen and gluconeogenic sources to postabsorptive GP between euglycemic cirrhotic subjects and healthy controls. Cirrhosis is associated with global changes in the contribution of substrates to whole-body energy production with a significant shift from carbohydrate to fat utilization [10]. Hepatic glycogen synthesis and hydrolysis are impaired, limiting the availability of dietary carbohydrates to the rest of the body while gluconeogenesis accounts for a larger fraction of postabsorptive GP. The contribution of glycogen to GP after an overnight fast was quantified in cirrhotic and healthy subjects by measuring ^2H-enrichment in position 5 relative to position 2 of glucose with GC-MS following ingestion of ^2H_2O [1,2]. In both studies, significantly lower contributions of glycogen to GP were found in cirrhotics compared to healthy subjects. Therefore, as a primary aim, we sought to verify these observations by quantifying enrichment in positions 5 and 2 of plasma glucose in a similar experimental setting with the ^2H NMR procedure. Subsequently, further insight on the sources and mechanisms of GP were obtained from analysis of ^2H-enrichment in the other positions of plasma glucose.

2. Materials and methods

2.1. Human studies

All subjects were studied with a protocol approved by the University of Coimbra Hospital Ethics Committee. The data in this report were obtained from five healthy subjects and six patients with biopsy-confirmed cirrhosis that were candidates for liver transplantation. The healthy group consisted of three males and two females with a mean age of 25 ± 8 years (range 19–39 years) and mean weight of 68 ± 10 kg (range 54–78 kg). The patient group consisted of four females and two males with a mean age of 37 ± 13 years (range 22–64 years) and mean weight of 64 ± 14 kg (range 45–85 kg). The patients were diagnosed with cirrhosis by liver biopsy 6–12 months before the tracer measurements. None of the patients had hepatitis or other incurrent diseases. The two subjects diagnosed with alcoholic cirrhosis had abstained from alcohol for at least 12 months preceding the study. Etiologies, child classification and medications for the cirrhotic patients are summarized in Table 1.

Subjects began fasting at 20:00. During the fast, they ingested a quantity of ^2H_2O designed to achieve body water enrichment of ~ 0.5%. For each subject, the amount of 70%-enriched ^2H_2O required to achieve 0.5% body water enrichment was ~ 260 ml. To reduce the possibility of vertigo, this was divided into two portions of ~ 130 ml, each taken at 01:00 and 06:00 (5 and 10 h into the fast). To ameliorate the unusual taste of highly enriched ^2H_2O and to further reduce the risk of vertigo, each portion was diluted with ~ 200 ml of bottled spring water. Body water mass was assumed to be 60% of the total weight for men and 50% for women [4]. For the remainder of the study, all subjects ingested 0.5% ^2H_2O in bottled spring water ad libitum. At 11:00, 11:20, 11:40 and 12:00, 20 ml of blood was drawn

Table 1
Etiology and medication of the cirrhotic patients

Patient	Age/sex	Etiology	Child quotient	Medication
F	40/F	alcoholic cirrhosis	B	furosemide
G	52/F	primary biliary cirrhosis	B	ursodiol, furosemide
H	33/M	primary sclerosing cholangitis	B	ursodiol, furosemide
I	25/M	criptogenic cirrhosis	C	ursodiol, furosemide
J	64/F	alcoholic cirrhosis	B	furosemide
K	22/F	Wilson's disease	A	penicillamine

from a peripheral vein. Plasma glucose levels were measured from the 12:00 blood collection using a glucose assay kit (Sigma Chemical Company). Urine was also collected from 10:30 to 12:00 at which point the study was concluded.

2.2. Sample processing

Blood was stored at 4 °C and centrifuged within 2 h from being drawn. For some samples, the plasma supernatant protein was precipitated by centrifugation following addition of 1/10th the plasma volume of 70% perchloric acid. The supernatant was neutralized with KOH and lyophilized after $KClO_4$ was removed by centrifugation. For the remaining samples, plasma protein was precipitated by the addition of 4 volumes of methanol per volume of plasma and the supernatant was recovered by filtration via a Buchner funnel. The methanol was removed by rotary evaporation at 40 °C; after which the remaining aqueous fraction was removed by lyophilization. For conversion of plasma glucose to the monoacetone derivative, the lyophilized extracts were treated with 3–20-ml anhydrous acetone enriched to 0.15% with acetone-d_6 to which concentrated sulfuric acid (4% v/v) was added. The solution was quickly cooled to room temperature, and MAG was prepared using the procedure of Snowden [11]. In the final stages of the preparation, the solvent was removed by rotary evaporation and MAG was extracted from the dried residue with a few milliliters of boiling ethyl acetate or 1 ml of 95% acetonitrile:5% H_2O. The residue from this extraction was dissolved in 0.6 ml of 90% acetonitrile:10% water, made basic with a few grains of sodium carbonate and placed in a 5-mm NMR tube for analysis [7].

2.3. NMR spectroscopy

NMR spectra were acquired at 11.75 T with a Varian Unity 500 system equipped with a 5-mm broadband "switchable" probe with z-gradient (Varian, Palo Alto, CA). ^2H was observed with the 'broadband' coil tuned to the ^2H carrier frequency. For this particular probe, detuning the lock coil beforehand resulted in a substantial reduction of the 90° pulse width for ^2H on the broadband coil (~ 30 to 19 μs). Shimming was performed on the ^1H signal using both the Varian automated gradient shimming routine and manual adjustment in response to the ^1H-linewidths of selected resonances. Proton-decoupled ^2H NMR spectra of MAG derivatives were obtained without lock at 50 °C using a 90° pulse and a 1.2-s acquisition time. Absolute enrichment of glucose hydrogen 2 in selected MAG samples was obtained by addition of a ^2H-formate standard of known enrichment to the NMR sample [12]. The number of free-induction decays ranged from 30,000 to 60,000 (10–20 h). Fully relaxed ^1H NMR spectra were obtained under the same conditions with pre-saturation of the acetonitrile signal using a pulse width of 45° and delay of 16.5 s. Four to sixteen free-induction decays were acquired for each spectrum. Deuterium enrichment of urine and plasma water was analyzed by ^2H NMR as previously described [13]. A pulse width of 21° and delay of 12.8 s were used to obtain fully relaxed ^2H NMR signals. The number of free-induction decays acquired for each spectrum ranged from 40 to 100. At least two ^2H_2O enrichment standards were run during each NMR session, in addition to a separate calibration curve generated with ^2H_2O enrichment standards ranging from 0.2% to 1.0%. The relative areas of selected peaks in both ^1H and ^2H NMR spectra were analyzed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR Inc., Fremont CA). ^2H_2O was obtained from Cambridge Isotopes, Cambridge, MA and Eurisotop, Gif-sur-Yvette, France.

2.4. Calculating the contribution of glycogen and gluconeogenic substrates to GP

The percent contribution of glycogen and gluconeogenic substrates [glycerol + phosphoenolpyruvate (PEP)] to GP was estimated directly from the ratio of deuterium signal intensities of hydrogens 2 and 5 of the ^2H NMR spectrum of MAG [9,12].

$$\text{Percent GP from glycogen} = 1 - (\text{signal } 5/\text{signal } 2) \times 100 \quad (1)$$

$$\text{Percent GP from gluconeogenesis} = (\text{signal } 5/\text{signal } 2) \times 100 \quad (2)$$

The gluconeogenic contribution to GP was resolved into contributions from glycerol and PEP derived from the hepatic Krebs cycle based on the intensity of the hydrogen 6S signal relative to those of 5 and 2 [9,12].

$$\text{Percent of GP from Krebs cycle (PEP)} = (\text{signal } 6S/\text{signal } 2) \times 100 \quad (3)$$

$$\text{Percent of GP from glycerol} = [(\text{signal } 5 - \text{signal } 6S)/\text{signal } 2] \times 100 \quad (4)$$

2.5. Data presentation and statistics

Unless otherwise stated, ^2H-enrichment data for each subject represent the mean analysis of three individual blood samples, each of which was derivatized to MAG and analyzed by ^2H NMR. Likewise, the measurement of body water enrichment was performed in triplicate for each subject. Statistical analysis of the data was performed with Microsoft Excel.

3. Results

Fasting plasma glucose levels of controls and patients were comparable with a mean of 87 ± 7 mg/dl (range 74–95 mg/dl) for the controls and 87 ± 24 mg/dl (range 57–117 mg/dl) for the patients. Mean body water enrichment levels were also similar: 0.50 ± 0.11% with a range of 0.41–0.70% for controls, and 0.48 ± 0.12% with a range of 0.27–0.60% for the patients. Enrichment of glucose hydrogen 2, determined from single MAG samples from each subject, was 98 ± 9% and 102 ± 9% that of body water for controls and cirrhotic subjects, respectively. This indicates complete equilibration of ^{2}H between body water and glucose hydrogen 2. Good quality ^{2}H NMR spectra of MAG prepared from single 20-ml blood samples were obtained for most of the subjects, as shown in Fig. 1. The coefficient of variation for relative ^{2}H-enrichment values obtained from three individual measurements in a single subject ranged from 7% to 12%. In two control subjects (B and D), yields of MAG from individual blood samples were low, resulting in spectra with poor signal-to-noise ratios. For each subject, two to three MAG samples were pooled together to obtain a single ^{2}H NMR spectrum.

In the ^{2}H NMR spectrum of MAG, the intensity of each NMR signal is a direct measure of the level of ^{2}H-enrichment at that particular site. Quantification of gluconeogenic and glycogenolytic fluxes by Landau's method requires measurement of the relative enrichment in positions 2 and 5 of glucose. For the range of glucose enrichment levels attained (~ 0.2–0.6%), the contribution of natural-abundance ^{2}H is too small to significantly perturb the enrichment measurements. ^{2}H NMR spectra of MAG derived from healthy controls after 16 h of fasting (Fig. 1A–E) are consistent with recent ^{2}H NMR measurements of MAG, prepared from plasma glucose of healthy individuals who had ingested similar levels of ^2H_2O and had fasted for a comparable period. Hydrogen 2 produced the most intense signal, reflecting maximal enrichment of glucose hydrogen 2 due to its quantitative exchange with the ^{2}H-enriched body water hydrogen. Signals from the other MAG hydrogens were ~ 40–60% the intensity of hydrogen 2, reflecting dilution of these labeling sites by glucose generated from glycogenolysis. Under the study conditions, glucose molecules derived via the direct pathway from glycogen are assumed to have no enrichment from ^2H_2O apart from hydrogen 2, which is assumed to be fully exchanged with the hydrogens of water and is therefore enriched to the same level as body water. Relative ^{2}H-enrichments from all seven aliphatic hydrogens of the glucose skeleton were directly obtained from the ^{2}H-signal area ratios of MAG and the values are shown in Table 2. Enrichment of hydrogen 5 was about half that of hydrogen 2, hence the total contribution of gluconeogenic substrates to GP (54 ± 4%) was almost matched by that of glycogen (46 ± 4%). These observations are in good agreement with both GC-MS and NMR measurements of healthy individuals given similar dosages of ^2H_2O and fasted for similar intervals [1,2,9,14]. The relative enrichment of hydrogen 6S approached that of hydrogen 5, suggesting that PEP was the principal gluconeogenic substrate with only minor contributions from glycerol (13% of

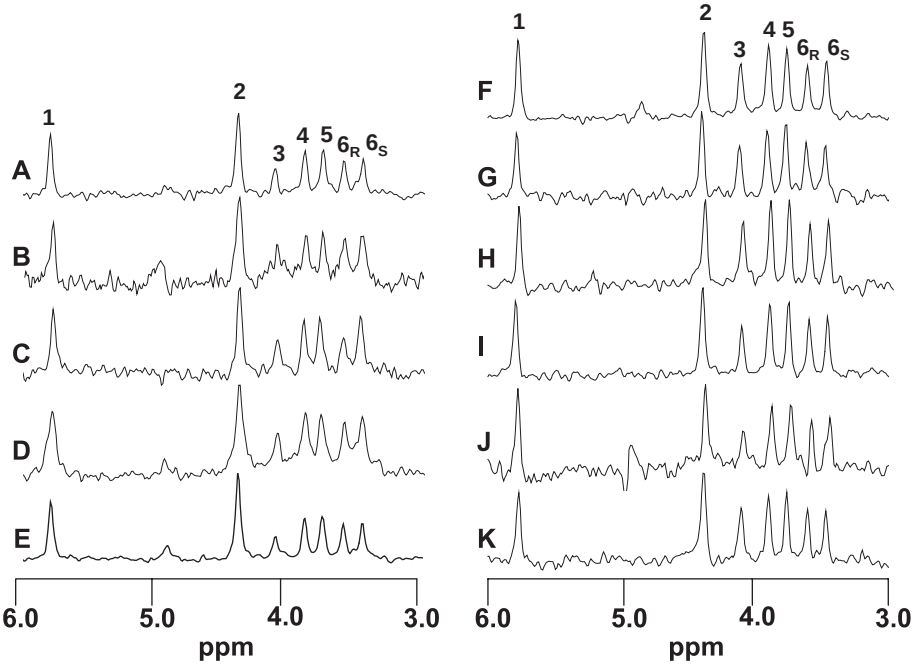

Fig. 1. ^{2}H NMR spectra of monoacetone glucose sample prepared from plasma glucose obtained blood samples of healthy (A–E) and cirrhotic (F–K) subjects. Spectra represent single 20-ml blood samples, with the exception of spectra B and D, which represent two and three pooled samples from each subject, respectively.

gluconeogenic contributions and 7 ± 6% of GP). Consistent with recent NMR data [9], relative enrichment of hydrogen 4 was essentially equal to that of hydrogen 5 while the relative enrichment of hydrogen 3 was significantly less than that of hydrogen 5. The 6R and 6S hydrogens of MAG had similar levels of enrichment, indicating that the methylene hydrogens of PEP and Krebs cycle precursors had been uniformly enriched from ^2H_2O. Finally, enrichment of hydrogen 1 was significantly higher than hydrogen 6R or 6S, indicating exchange between hydrogen 1 of G-6-P and that of body water via hexose phosphate isomerases [15].

^{2}H NMR spectra of MAG from cirrhotic subjects were noticeably different from those of controls (Fig. 1F–K). Typically, the intensities of hydrogens 1, 4 and 5 signals approached that of hydrogen 2, while the hydrogen 3 and 6 signals were less intense. Moreover, the difference in intensity between the signal of hydrogen 5 and that of 6S was typically greater in MAG spectra of cirrhotics compared to those from controls. With the exception of hydrogen 6S, relative ^{2}H enrichments were significantly higher than the corresponding values for controls (Table 2). Not surprisingly, estimates of glycogenolysis and gluconeogenesis derived from spectra of cirrhotics showed significant differences from those of healthy controls (see Fig. 2). For the cirrhotic group, the majority of glucose was derived from gluconeogenesis (78 ± 7%) with only minor contributions from glycogenolysis (22 ± 7%).

Table 2
^{2}H relative enrichments of hydrogens 1–6 of plasma glucose from healthy (A–E) and cirrhotic subjects (F–K) derived from the relative areas of the ^{2}H NMR signals

	Relative ^{2}H enrichment						
	1	2	3	4	5	6R	6S
Controls							
A	68	100	31	48	51	36	40
B*	56	100	48	55	57	59	56
C	74	100	42	54	59	41	58
D*	79	100	40	55	55	40	45
E	62	100	23	44	50	36	36
Mean	68[ab]		37[cd]	51[e]	54[e]	42[b]	47
S.D.	9		10	5	4	10	10
Cirrhotics							
F	84	100	65	79	77	60	58
G	77	100	61	78	80	59	63
H	88	100	88	92	89	69	76
I	74	100	51	75	78	43	49
J	90	100	57	73	78	60	55
K	71	100	48	66	66	49	50
Mean	81[f]		62[g]	77	78	57	59
S.D.	8		14	9	7	9	10

Enrichment of position 2 is arbitrarily set to 100.

* Values derived from a single NMR spectrum of two or three pooled MAG samples.

[a] Significantly different from hydrogen 6S of control ($P<0.01$).

[b] Significantly different from the corresponding hydrogen of cirrhotic ($P<0.05$).

[c] Significantly different from hydrogen 5 of control ($P<0.02$).

[d] Significantly different from the corresponding hydrogen of cirrhotic ($P<0.01$).

[e] Significantly different from the corresponding hydrogen of cirrhotic ($P<0.001$).

[f] Significantly different from hydrogen 6S of cirrhotic ($P<0.005$).

[g] Significantly different from hydrogen 5 of cirrhotic ($P<0.04$).

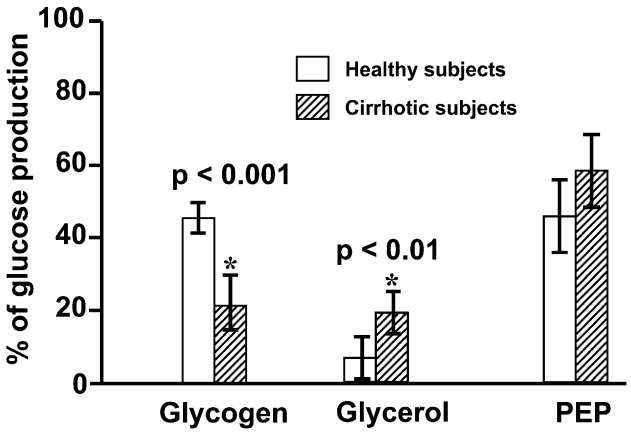

Fig. 2. Percentage contribution of glycogen, glycerol and PEP to glucose production in healthy and cirrhotic subjects.

In accordance with the larger difference between hydrogen 5 and 6S enrichments, the estimated contribution of glycerol was significantly higher in cirrhotics compared with healthy subjects, accounting for about one quarter of gluconeogenic output and 19 ± 6% of GP. However, this estimate assumes full equilibration between the hydrogens of water and those of hepatic oxaloacetate (OAA). Since this was not verified in our study, we cannot exclude the possibility that part of the difference between hydrogen 5 and 6S enrichments could be due to incomplete exchange of water and OAA hydrogens. These conditions will result in overestimates of the fractional contribution of glycerol to gluconeogenesis with the PEP contribution being correspondingly underestimated. Furthermore, the possibility that the contribution of glycerol to the glyceraldehyde-3-phosphate (G3P) and 1-*R*-dihydroxyacetone phosphate (DHAP) moieties of glucose may not be equal places further uncertainties on the measurement (see discussion). Given these uncertainties and the fact that the hydrogen 6S to hydrogen 2 enrichment ratios was not significantly higher in cirrhotics compared to controls ($P=0.08$), the hydrogen 5:2 rather than the 6/2 enrichment ratio appears to be the more reliable marker of changes in contributions of gluconeogenic and glycogenolytic fluxes to GP.

4. Discussion

We demonstrated that ^{2}H-enrichment in all sites of plasma glucose can be routinely determined by ^{2}H NMR following ingestion of ^2H_2O to 0.5% body water. Significant differences in glucose ^{2}H-enrichment patterns were found between healthy and cirrhotic subjects, reflecting a significant decrease in the contribution of glycogen to GP as a

result of cirrhosis. The small number of subjects and the diversity of disease etiologies and extent of liver damage as defined by the Child Score preclude any specific conclusions on the sensitivity of this measurement to the severity of cirrhosis to be made. Nonetheless, the fact that all patients presented elevated gluconeogenic contributions suggests that the changes in relative glycogenolytic and gluconeogenic fluxes occur early rather than late in the progression of cirrhosis. This observation is consistent with recent ^2H_2O studies of cirrhotic subjects [1,2].

4.1. Estimates of gluconeogenesis and glycogenolysis in cirrhosis

Our results are in good agreement with two recent studies where GC-MS analysis of plasma glucose was used to compare the contribution of gluconeogenesis to GP in cirrhotic and healthy subjects using ^2H_2O ingestion protocols and fasting times similar to those of our study. In the study of Petersen et al. [1], the contribution of gluconeogenesis to GP after 18.5 h of fasting was ~70% in cirrhotic patients compared to 55% in healthy controls. Bugianesi et al. [2] reported a 74% contribution of gluconeogenesis to GP in cirrhotic subjects compared to 56% in healthy controls after 17 h of fasting. The dominance of the gluconeogenic contribution to GP in overnight-fasted cirrhotic resembles that found in a healthy subject fasted for an extended period (<24 h) [5,14]. However, the underlying causes appear to be quite different. Healthy individuals that have fasted for 24 h or more have a high gluconeogenic and low glycogenolytic contribution to GP primarily because of severely depleted hepatic glycogen reserves. However, hepatic glycogen levels of overnight-fasted cirrhotic subjects are not depleted to the same extent [10] and would therefore be expected to sustain higher rates of glycogenolysis than those observed. Direct quantification of hepatic glycogen concentrations by ^{13}C NMR revealed major differences in hepatic glycogen levels between cirrhotic and healthy subjects after 5 h of fasting, but no significant differences after 14 h of fasting [1]. Despite the similar hepatic glycogen levels in the two groups at this stage of fasting, the fractional contribution of glycogenolysis to GP, as measured by the ^2H_2O method, was significantly less in the cirrhotic subjects. These findings suggest that the low contribution of glycogenolysis to GP in cirrhosis may be due to attenuated glycogen degradation rather than a scarcity of hepatic glycogen. The sensitivity of glycogenolysis to glucagon was found to be lower in cirrhotic subjects compared to healthy controls, the effect being independent of hepatic glycogen levels [2]. Meanwhile, gluconeogenesis may be stimulated because of increased lipolysis, which releases more glycerol and free-fatty acids (FFA) for hepatic consumption. Glycerol contributes directly to the gluconeogenic triose phosphate pool while FFA oxidation promotes the anaplerotic utilization of pyruvate via pyruvate carboxylase while inhibiting pyruvate dehydrogenase activity. Thus, anaplerotic flux and net export of PEP carbons for gluconeogenesis is increased, while FFA oxidation supplies the ATP and NADH requirements.

Finally, the effect of the indirect pathway of glycogen metabolism on the interpretation of the plasma glucose ^2H-enrichment data needs to be considered. Glycogen that is converted to glucose via this route will incorporate ^2H in position 5 and will augment the estimate of fractional gluconeogenesis. Therefore, the increased 5/2 ^2H-enrichment ratio of glucose observed in the cirrhotic patients could be explained by a redistribution of glycogen degradation from the direct pathway to the indirect pathway without any change in the absolute rate of hepatic glycogenolysis. In cirrhotic subjects, the absolute rate of glycogenolysis was measured directly by ^{13}C NMR of hepatic glycogen and was found to be significantly lower than that of healthy controls [1]. Moreover, these estimates were in agreement with absolute rates of glycogenolysis obtained by integration of the ^2H_2O and GP measurements from the same subjects [1]. This supports the hypothesis that the 5/2 ^2H-enrichment ratio of glucose provides reasonable estimates of the contribution of glycogenolysis and gluconeogenesis to GP and that the direct pathway accounts for the bulk of postabsorptive glycogen conversion to glucose in both healthy and cirrhotic subjects. For other physiological or pathophysiological states where there is no information on the mode of glycogen degradation, the 5/2 ^2H-enrichment ratio of glucose should be assumed to represent the upper limit of fractional gluconeogenesis.

4.2. New ^2H-enrichment information of glucose

The NMR analysis provides new information on glucose enrichment from ^2H_2O. For the first time, the ^2H-enrichment distribution in hydrogens 3, 4 and 5 of glucose from ^2H_2O is fully resolved. Previously, analysis of ^3H labeling patterns in this fragment could not be fully resolved because of the lack of simple and specific degradation methods for quantifying labeling in the individual sites [16,17]. GC-MS methods for analyzing ^2H enrichment levels in these sites are based on quantifying the difference in excess enrichment between different fragments of glucose and are prone to significant errors at enrichment levels below 5% [18].

From the ^2H NMR analysis, relative enrichment of glucose hydrogen 4 was found to be equal to that of hydrogen 5 for all subjects. Hydrogen 4 of gluconeogenic glucose is derived from hydrogen 1 of G3P. Transfer of ^2H-enrichment from water to this hydrogen can occur via reduction of 3-phosphoglycerate following enrichment of NADH from ^2H_2O. Note that this mechanism is specific for triose units derived from PEP and would not label G3P derived from glycerol. Exchange of G3P hydrogen 1 with water, catalyzed by aldolase, provides a second mechanism for ^2H-enrichment [19]. In this case, G3P molecules derived from both PEP and glycerol will be enriched with deuterium. If aldolase exchange is quantitative, hydrogen 1 of

G3P will be enriched to the same level as body water. Given that hydrogen 2 of G3P is also enriched to the same level as body water [14], then enrichment of hydrogens 4 and 5 of glucose will also be equal. This is consistent with our experimental findings. Whether hydrogen 4 and 5 enrichments are equal under isotopic non-steady state conditions is not known and will require further study. In fasted rats, plasma glucose recovered immediately after a 2.5-h infusion of ^2H_2O had equal enrichment in hydrogens 4 and 5 [20].

Enrichment of hydrogen 3 was significantly less than that of hydrogen 5, indicating that enrichment of the DHAP hydrogen (the precursor of glucose hydrogen 3) was less than that of body water. This finding is in agreement with recent NMR measurements of glucose enrichment from ^2H_2O in healthy subjects [9]. These observations could reflect the discrimination of ^2H-incorporation from water into the 1*R* site of DHAP by triose phosphate isomerase. Alternatively, they can be explained by the incomplete exchange of G3P and DHAP via triose phosphate isomerase. Such conditions result in an excess of G3P over DHAP, requiring the input of DHAP from another source, presumably glycerol. Numerous reports of labeling distributions in the triose moieties of glucose from both ^{13}C- and ^{14}C-tracers support the latter postulate, notably the study of Ekberg et al. [21]. Under the conditions of our ^2H_2O study, glycerol is largely unenriched; hence its incorporation into glucose via DHAP will dilute the enrichment at position 3 of glucose. Note that this dilution by glycerol is not reflected in positions 1 and 2 of glucose because of further exchanges of these hydrogens with those of water at the hexose phosphate level [5,15]. Incomplete exchange of triose phosphates via triose phosphate isomerase has significant implications on the estimate of glycerol gluconeogenesis from ^2H_2O. The measurement, based on differences between glucose hydrogen 5 and 6 enrichments [14], reports glycerol and PEP contributions to the triose moiety of glucose derived from G3P. Extending this analysis to glucose requires that exchange of G3P and DHAP is complete so that both halves of the glucose molecule are derived from the same proportion of glycerol and PEP precursors. When triose phosphate isomerase exchange is incomplete, we conclude that estimates of glycerol gluconeogenesis will be improved by evaluating enrichment information from both halves of the glucose molecule and by combining measurements of glucose ^{13}C-enrichment from ^{13}C-enriched triose phosphate precursors (i.e. ^{13}C-glycerol or ^{13}C-lactate) with ^2H-incorporation from ^2H_2O. As recently demonstrated, NMR can be used to resolve and quantify excess enrichment from a combination of ^2H_2O and ^{13}C tracers [9].

Enrichment of the hydrogen 6 sites of glucose by ^2H is mediated by several exchange mechanisms at the level of OAA and pyruvate. Relative enrichment of the OAA methylene sites is conserved in the methylene moieties of PEP and subsequently the hydrogen 6 sites of glucose. Since enrichment of the R and S methylene hydrogens of OAA occur by different exchange mechanisms,[1] the possibility exists that they (and by extension, the 6R and 6S hydrogen of glucose) might be enriched to different levels. The NMR measurements indicate that the various exchanges and recycling pathways at the level of pyruvate and the Krebs cycle resulted in uniform enrichment of the OAA methylene sites. This enrichment pattern is consistent with, but is insufficient proof for[2] complete exchange of OAA and water hydrogens. Given the uniform enrichment of the glucose 6R and 6S hydrogens, the average enrichment of hydrogen 6 relative to hydrogen 2 (H6/H2) derived by the GC-MS procedure and the enrichment of hydrogen 6S relative to hydrogen 2 (H6S/H2) measured by NMR should provide consistent values. In the study of healthy individuals by Landau et al. [14], H6/H2 was 0.33 at 14 h and 0.47 at 22 h of fasting. The ^2H_2O study by Buiganeisi et al. [2] reported H6/H2 of 0.38 for healthy subjects fasted for 16–17 h. The H6S/H2 for healthy subjects in this report was 0.47 while the average relative enrichment of both hydrogen 6 sites was 0.45. In the earlier NMR study, H6S/H2 was 0.45 for subjects fasted for 16–18 h [9]. The NMR values fall at the high end of the range of MS measurements for healthy individuals fasted for 14–18 h. Whether the two methods give significantly different estimates of hydrogen 6 enrichment is unclear, given the large variation in gluconeogenesis within a group of healthy subjects and the relatively small number of studies available for comparison. A more definitive comparison of MS and NMR enrichment estimates would involve applying both methods to a common set of plasma glucose samples.

4.3. Analytical considerations

In comparison with current GC-MS methods, the NMR approach is very simple and provides the entire ^2H-enrichment distribution of glucose in a single measurement. Moreover, the procedure is amenable to extensive systemization with modern NMR instrumentation and data analysis software. As illustrated by this study, quantification of ^2H-enrichment by ^2H NMR from reasonably small blood volumes with body water enrichment of 0.5% is feasible with conventional NMR probes and an 11.75-T magnet. The coefficients of variation from repeated analysis of individuals in our study (7–12%, $n=3$) were higher than those reported in a recent GC-MS study (1.0–2.6%, $n=5$) [5]. Much of the variability in the NMR analysis is probably attributable to the level of noise in the free-induction decay. Therefore, the reproducibility of NMR analysis at 11.75 T

[1] FUM → *R*-[3-^{2}H]MAL → *R*-[3-^{2}H]OAA → (*Z*)-[3-^{2}H]PEP → *S*-[3-^{2}H]PGA → → *S*-[6-^{2}H]glucose. [3-^{2}H]PYR → *R*,***S***-[3-^{2}H]OAA → (***E***,*Z*)-[3-^{2}H]PEP → ***R***,*S*-[3-^{2}H]PGA → → ***R***,*S*-[6-^{2}H]glucose. Enrichment of pyruvate is conserved in the *S* hydrogen of OAA irrespective of OAA↔fumarate exchange.

[2] This would require direct quantification of 3R and 3S enrichment of hepatic OAA relative to that of body water.

should benefit from the significant improvements in sensitivity afforded by smaller-volume probes or cryo-probes.

In conclusion, the NMR measurement provides a complete analysis of glucose ^{2}H-enrichment from ^2H_2O in the clinical setting. The analysis identified abnormal glycogenolytic and gluconeogenic contributions to GP in euglycemic cirrhotic patients. The simplicity of the method and the fact that it can be performed on a conventional NMR system should allow Landau's ^2H_2O ingestion measurement to be more widely and routinely applied.

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